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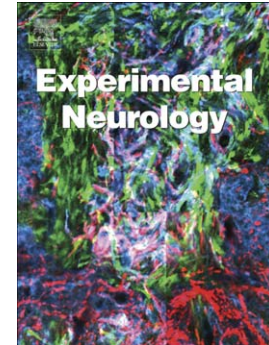
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Intranasal C3a treatment ameliorates cognitive impairment in a mouse model of neonatal hypoxic-ischemic brain injury

Javier Morán¹, Anna Stokowska¹, Frederik R. Walker², Carina Mallard³, Henrik Hagberg^{3,4,5}, Marcela Pekna^{1,6,7*}

¹Center for Brain Repair and Rehabilitation, Department of Clinical Neuroscience, Institute of Neuroscience and Physiology, Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden

²School of Biomedical Sciences and Pharmacy, University of Newcastle, New South Wales, Australia.

³Perinatal Center, Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

⁴Centre for the Developing Brain, King's College, London, UK

⁵Department of Obstetrics and Gynecology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

⁶Florey Institute of Neuroscience and Mental Health, Parkville, Victoria, Australia.

⁷Hunter Medical Research Institute, University of Newcastle, New South Wales, Australia.

***Corresponding author:**

Marcela Pekna, MD, PhD

Institute of Neuroscience and Physiology

Dept. of Clinical Neuroscience

The Sahlgrenska Academy at University of Gothenburg

Box 440, SE-405 30 Gothenburg

Sweden

Tel: +46-31-786 3581

Fax: +46-31-416 108

E-mail: Marcela.Pekna@neuro.gu.se

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Summary

Perinatal asphyxia-induced brain injury is often associated with irreversible neurological complications such as intellectual disability and cerebral palsy but available therapies are limited. Novel neuroprotective therapies as well as approaches stimulating neural plasticity mechanism that can compensate for cell death after hypoxia-ischemia (HI) are urgently needed. We previously reported that single i.c.v. injection of complement-derived peptide C3a 1 h after HI induction prevented HI-induced cognitive impairment when mice were tested as adults. Here, we tested the effects of intranasal treatment with C3a on HI-induced cognitive deficit. Using the object recognition test, we found that intranasal C3a treated mice were protected from HI-induced impairment of memory function assessed 6 weeks after HI induction. C3a treatment ameliorated HI-induced reactive gliosis in the hippocampus, while it did not affect the extent of hippocampal tissue loss, neuronal cell density, expression of the pan-synaptic marker synapsin I or the expression of growth associated protein 43. In conclusion, our results reveal that brief pharmacological treatment with C3a using a clinically feasible non-invasive mode of administration ameliorates HI-induced cognitive impairment. Intranasal administration is a plausible route to deliver C3a into the brain of asphyxiated infants at high risk of developing hypoxic-ischemic encephalopathy.

Keywords: neonatal encephalopathy / hypoxia-ischemia / complement system / behavioral deficit / reactive gliosis

Abbreviations

HI, hypoxia-ischemia; HIE, hypoxic–ischemic encephalopathy; C3aR, C3a receptor; EBM, Experimental Biomedicine; P, postnatal day; PBS, phosphate buffered saline; ORT, object recognition test; ISI, intersession interval; STM, short-term memory; LTM, Long-term memory; PFA, paraformaldehyde; NeuN, Neuronal nuclei; GAP-43, growth associated protein 43; SYN, synapsin I; VGLUT1, vesicular glutamate transporter 1; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adapter molecule 1; CA, cornu ammonis; DG, dentate gyrus; PBS-T, PBS with 0.05% Tween 20; DAB, diaminobenzidine; ANOVA, Analysis of Variance.

Introduction

Neonatal hypoxic–ischemic encephalopathy (HIE) due to perinatal asphyxia is the leading cause of neurological injury resulting from birth complications. It is caused by the disruption of blood flow and oxygen delivery to the brain prior to or during delivery and occurs in 1-3 of 1000 live term births (Kurinczuk et al., 2010). Recent advances in critical care have improved the survival of infants suffering from HIE, but approximately 50% of survivors will develop complications such as intellectual disability and cerebral palsy (Mwaniki et al., 2012). Therapeutic hypothermia of children with HIE is a clinically accepted therapy that reduces by 12% the number of children with disabilities at 2 years of age (Edwards et al., 2010). Inflammation is a critical contributor to both normal development and injury outcome in the immature brain; depending on the timing and context, inflammation can prime the brain for injury or be neuroprotective (Hagberg et al., 2015).

Although best known for its role in the elimination of pathogenic bacteria, complement has also other functions such as the initiation of inflammation and the regulation of antibody production. Research during the past 10 years has shown that complement is a major regulator of brain plasticity and function in the healthy as well as diseased brain. Complement regulates the number of synapses during CNS development (Perez-Alcazar et al., 2014; Schafer et al., 2012; Stevens et al., 2007), promotes neurogenesis in the adult mammalian CNS (Rahpeymai et al., 2006) and the complement activation-derived peptide C3a stimulates neurite outgrowth as well as neuronal differentiation of neural progenitor cells *in vitro* (Shinjyo et al., 2009). C3 is up-regulated in sprouting neurons isolated from rat cortex after ischemic stroke (Li et al., 2010) and C3a promotes astrocyte survival in response to ischemia (Shinjyo et al., 2016). Using transgenic mice over-expressing

C3a in the brain and injection of C3a into the brain ventricles of control and C3a receptor (C3aR) deficient mice 1 hour after HI, we showed that C3a, acting through C3aR, is protective against neonatal HI-induced tissue loss and cognitive impairment (Järlestedt et al., 2013).

Intraventricular administration of drugs is not clinically feasible and systemic administration of C3a carries a risk of serious adverse reactions including anaphylaxis (Finkelman et al., 2016). In addition, the availability in the CNS of systemically administered C3a would be limited due its rapid inactivation by serum carboxypeptidases (Bokisch and Muller-Eberhard, 1970) as well as by the blood-brain barrier. Intranasal administration permits peptides to bypass the periphery and the blood-brain barrier, rapidly reaching the brain and entering the cerebrospinal fluid. Molecules delivered intranasally use extracellular bulk flow transport along olfactory and trigeminal perivascular channels and possibly also axonal transport (Bahadur and Pathak, 2012). Proteins with size of up to 20 kDa, including insulin-like growth factor 1, nerve growth factor and epidermal growth factor, have been successfully delivered to the brain using this method (De Rosa et al., 2005; Lin et al., 2009; Scafidi et al., 2014). Intranasal administration thus appears as an attractive, clinically highly relevant and non-invasive method of therapeutic delivery of C3a to the brain. We therefore sought to determine whether the HI-induced cognitive deficit could be reversed by brief intranasal treatment with C3a.

Materials and methods

Animals

Subjects were male C57BL6/CNr mice (Charles River Laboratories, Sultzfield, Germany). The local Animal Ethics Committee in Gothenburg (308-2012; 41-2015) approved all animal experiments and mice were housed at Experimental Biomedicine (EBM), Sahlgrenska Academy, University of Gothenburg. Mice were kept under standard conditions of temperature (20°C), and relative humidity (45%) and on an artificial light-dark cycle of 12 h (lights on at 06:00). Food and water were available *ad libitum*.

Experimental design

Two groups of male mice were subjected to neonatal HI injury and intranasally treated for 3 days with either C3a (HI-C3a, n=18) or PBS (HI-PBS, n=18). A control group of sham-operated animals was treated with PBS (SHAM-PBS, n=19). These mice were tested in an open field and object recognition test at P50-P54 and brains were collected at P55 (Fig. 1A).

HI injury induction

Neonatal HI injury was induced on postnatal day 9 (P9), as previously described with modifications for mice (Hedtjörn et al., 2002; Järlestedt et al., 2013; Rice et al., 1981; Sheldon et al., 1998). Mice were anesthetized with 3.5% isoflurane (Baxter Medical, Kista, Sweden) for induction and 1.5% thereafter, in 1:1 oxygen and nitrous oxide. The left common carotid artery was dissected and

permanently ligated with a prolene suture. The incision was closed and infiltrated with lidocaine (Xylocain®, Astra Zeneca, Gothenburg, Sweden). Mice were returned to the dam for 1 hour and then placed in a chamber with humidified air at 36° C for 10 min, then exposed to humidified 10% oxygen in nitrogen for 30 min at 36° C, and then kept in humidified air at 36° C for 10 min before being returned to the dam. Sham animals were subjected to an incision in the neck on P9. These pups were also removed from the dam for the time duration that injured animals stayed in the chamber, but remained instead in a warming tray at 36°C under normal oxygen conditions. At postnatal day 21 (P21) mice were weaned and group housed with same sex littermates. Given that this moderate HI injury affecting ipsilesional hippocampus and amygdala leads to persistent cognitive impairment without any apparent deficit motor function (Järlestedt et al., 2013), this model is particularly suitable for selective assessment of the effect of therapeutic interventions on learning and memory.

Intranasal C3a administration

Purified human C3a (Complement Technology Inc., Tyler, TX, USA) was diluted in sterile phosphate buffered saline (PBS) to a concentration of 200 nM, and a total of 8 µl, i.e. 1.6 pmol (4 µl /nostril; corresponding to ca. 2.56 µg/kg body weight) of peptide solution or PBS was given intranasally to awake and hand-restrained mice held in a supine position. Solutions were administered through a pipette tip, drop-wise in 2 µl-portions divided by 1 min intervals to allow for absorption. This method of administration to one nostril at a time does not affect breathing. C3a or PBS was given every 24 hours for three days starting 1 h after HI induction, i.e. between P9 and P11, respectively. Mice in each litter were randomly

assigned to C3a or PBS treatment. Sham animals received PBS. The investigators carrying out behavioral studies and analyzing data were blinded to treatment group.

Behavioral analysis

Object recognition test

The object recognition test (ORT) is based on the innate preference of mice to explore a novel object rather than a familiar one. Therefore, animals that remember the familiar object will spend more time exploring the novel object (Leger et al., 2013). From 3 days before starting the test, mice were daily handled for 2 min to minimize the possible stress due the researcher interaction. All experiments were performed at the same time of the day, between 9 am and 5 pm, and inside of a plastic box of 50x50x50 cm dimensions where the light intensity was dim and equal in all parts of the apparatus. Before being placed in the arena, mice had a 60 min habituation period in the behavioral room. Prior to the familiarization session, during which two identical objects were introduced, mice were habituated to the apparatus for 10 min during 3 consecutive days. Activity on the first habituation day was recorded as an open field test to assess locomotor and exploratory activities. During the familiarization session, two identical 250 ml bottles filled with shredded paper were placed at an equal distance from the arena walls (approximately 5 cm). Between mice, the apparatus and objects were cleaned with 50% ethanol to minimize olfactory cues. Animals were allowed to explore both objects for 10 min. A minimal exploration criterion of 20 s in total for both objects was used (Leger et al., 2013). Exploration was defined as directing the nose to the object at a distance <2 cm and/or touching the object with the nose or forepaws (Becerril-Ortega et al.,

2014). Six hours after the familiarization session, intersession interval (ISI)=6 h, animals were tested for short-term memory (STM). This involved replacing one familiar object with a novel object (T75 culture flask filled with sand) placed in the same position. Long-term memory (LTM) was tested 24 h after the familiarization (ISI=24 h) by replacing the novel object used during the STM testing with another novel object (Lego tower). Animals were allowed to explore both objects for 10 min with a minimal exploration criterion of 20 s for both objects, during the SMT and LTM testing. Animals that did not reach the 20 s criterion were excluded from the experiment. Mice were tracked by Viewer³ video tracking system (Biobserve, Bonn, Germany), and the data were presented as time spent exploring individual objects and total exploration time (s).

Open field

The activity of each animal on the first day of habituation to the object recognition test apparatus was recorded as an open field task to study locomotor and exploratory activities (Leke et al., 2012). Individual animals were placed in the square arena, and allowed to explore the apparatus for 10 min. The area was divided in 16 square zones, of which the four central squares (25% of the total area) were considered the central zone. The data were presented as average speed (cm/s), total activity (%), total locomotion time (s), total distance travelled (cm), distance travelled in the center (%), time spent in the center (%), number of rearing occurrences and number of grooming episodes.

Brain collection and processing

On P55, mice were deeply anesthetized with thiopental [Pentothal Sodium (0.01 ml/g body weight), Hospira, Illinois, USA)] and transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde (PFA) in 0.1 M PBS. Brains were removed and post-fixed in 4% PFA at 4°C for 24 h followed by 70% ethanol for 24 h. Tissue was processed using an automatic tissue processor (SAKURA Tissue Tek VIP 3000, Tournai, Belgium) and embedded in paraffin. Brains were cut at room temperature into 8- μ m serial coronal sections using a sliding microtome (Microm HM 450, Thermo Scientific, Massachusetts, USA), attached to silane coated slides and dried at RT.

Histomorphologic evaluation

For the histomorphologic evaluation, slides were incubated for 1 h at 65°C and stained with haematoxylin and eosin. A wide-field microscope (Nikon Eclipse 80i; Nikon Instruments Inc., Tokyo, Japan) equipped with a color camera (Nikon DXM 1200F) was used to obtain images of brain sections 208 μ m apart between -1.60mm and -2.02 mm relative to Bregma; 3 sections/mouse. ImageJ 1.46r software was used to trace around the ipsilesional and contralesional hippocampus and hemisphere. Volumes were calculated according to the Cavalieri's principle, where $V = \Sigma A \times P \times T$ (Svedin et al., 2007) and hippocampus volume / hemisphere volume ratio was calculated for each hemisphere.

Immunohistochemistry

Neuronal nuclei (NeuN), growth associated protein 43 (GAP-43), synapsin I (SYN), vesicular glutamate transporter 1 (VGLUT1), glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba-1) were visualized in the cornu ammonis (CA) and dentate gyrus (DG) of the dorsal hippocampus by immunohistochemistry. Briefly, following heat-induced antigen retrieval with 0.01 M citrate buffer (pH 6, 0.05% Tween 20) for 3x5 min, sections were washed 3x5 min with PBS-T (0.05% Tween 20), non-specific protein binding was reduced by incubation with blocking buffer [4% normal donkey serum (NeuN and Iba-1), 1% bovine serum albumin (GAP-43 and SYN), 3% normal goat serum (VGLUT1), 2% bovine serum albumin (GFAP) in PBS-T] for 1h at room temperature (RT). Tissue was then incubated with the primary antibody [anti-NeuN biotinylated (1:200, MAB 377B, Millipore, MA, USA), anti-GAP-43 (1:1000, MAB347, Millipore, MA, USA), anti-SYN (1:150, SC-7379, Santa Cruz, CA, USA), anti-VGLUT1 (1:500, AB5905, Millipore, MA, USA), anti-GFAP (1:200, Z0334, Dako, Stockholm, Sweden), anti-Iba-1 (1:500, 019-19741, Wako, Osaka, Japan)] in blocking buffer overnight at 4°C. One slide per staining batch was incubated only with blocking buffer without primary antibody and used as a negative control. Next, sections were washed 3x5 min with PBS-T and incubated with the secondary antibody [rabbit-anti mouse biotinylated Ig (1:200, GAP-43, E0354, Dako, Stockholm, Sweden), donkey-anti goat biotinylated Ig (1:200, SYN, 705065147, Jackson ImmunoResearch Inc., PA, USA), Alexa Fluor 488 goat-anti guinea pig Ig (1:500, VGLUT1, A11073, Molecular Probes, Oregon, USA), Alexa Fluor 488 goat-anti rabbit (1:2000, GFAP, A11034, Molecular Probes, Oregon, USA), donkey-anti rabbit biotinylated (1:500, Iba-1, 711-065-152, Jackson ImmunoResearch Inc., PA, USA), in blocking buffer for 1h at RT. After washing 3x5 min with PBS-T, sections were incubated with Streptavidin-Cy3 (1:100 (GAP-43,

SYN), 1:300 (NeuN), S6402, Sigma-Aldrich, Missouri, USA) in blocking buffer for 1h at RT. Then, sections were washed 3x5 min with PBS-T, mounted with ProLong Gold (P36931, Life Technologies, CA, USA) and cover slipped for 24h before being sealed with nail polish. For Iba-1 staining, following secondary antibody, sections were incubated with an avidin/biotin complex (VECTASTAIN® Elite ABC kit, PK-6100, Vector Laboratories Inc., CA, USA) followed by diaminobenzidine (DAB) Substrate Kit (SK-4100, Vector Laboratories Inc., CA, USA) according to manufacturer's instructions. Next, sections were washed 3x5min with PBS-T, dehydrated (70% EtOH 2 min, 95% EtOH 2 min, 100% EtOH 2 min) and cleared with xylene for 5 min. Slides were mounted with VectaMount medium (H-5000, Vector Laboratories Inc., CA, USA) and cover slipped.

NeuN positive cells, GFAP positive relative area, as well as GAP-43, SYN, and VGLUT1 positive puncta were counted by using MetaMorph software (ver. 7.8.6, Molecular Devices, CA, USA) on confocal images obtained with a 20x (NeuN and GFAP) or 63x (GAP-43, SYN, and VGLUT1) objective (Carl Zeiss LSM 700 Laser Scanning Microscope, Jena, Germany). Iba-1 positive cell somata were counted on bright field images obtained with a 20x objective (Nikon Eclipse 80i). The entire region of CA1, CA3 and DG was imaged for NeuN, GFAP and Iba-1 analysis, while stratum oriens of CA1 and molecular layer of DG were used for sections stained with GAP-43, SYN, and VGLUT1 antibodies. Three sections per animal (208 μm apart) were used for the analysis and the data were presented as either density (positive cells / μm^2) for NeuN and Iba-1 or density (positive puncta / μm^2) and mean area of the positive punctum (μm^2) for GAP-43, SYN, and VGLUT1. For GFAP, the data were presented as positive area relative to the total area (%).

Statistical analysis

Data were analyzed with IBM SPSS Statistics 20 (New York, USA) and GraphPad Prism 6.0f (GraphPad Software Inc., CA, USA). The Gaussian distribution of data was verified using the Kolmogorov-Smirnov test, the variance homogeneity was assessed using the Levene Test. For the analysis of the total exploration time during the object recognition test and the open field experiment, one-way Analysis of Variance (ANOVA) was used, followed by a Tukey's multiple comparisons post-hoc test. Two-way ANOVA, with a Tukey's multiple comparisons post-hoc test, was used to analyze time spent exploring individual objects during the object recognition test, as well as the histomorphologic data and image analysis data. Two-way repeated measures ANOVA with a Tukey's multiple comparisons post-hoc test were used to analyze the body weight. Data are presented as mean \pm SEM. P values < 0.05 were considered statistically significant.

Results

Intranasal C3a treatment ameliorates HI-induced cognitive impairment

We previously reported that single i.c.v. injection of C3a 1 h after HI induction prevented HI-induced cognitive impairment when mice were tested as adults (Järlestedt et al., 2013). To determine whether such a protective effect of C3a can be achieved by intranasal administration, mice received C3a or PBS once daily for 3 days starting 1 h after HI induction and short and long-term memory was assessed at P53-P54 using object recognition test (Fig. 1A). We found that in the familiarization phase the exploratory behavior was not altered by HI or C3a treatment (Fig. 1B-C). Although HI injury did not affect the short-term memory, as assessed at 6 h after the familiarization, the C3a treated mice spent more time exploring the novel object compared with the PBS-treated mice post-HI ($p < 0.01$, Fig. 1D), which was also reflected in longer total object exploration time ($p < 0.05$, Fig. 1E). When exploratory behavior was tested 24 h after familiarization, the mice treated with PBS post-HI spent comparable time exploring the novel and familiar objects. In contrast, the sham-operated and C3a treated mice spent significantly longer time exploring the novel compared to familiar object ($p < 0.001$ and $p < 0.05$, respectively; Fig. 1F). Further, the total object exploration time of C3a treated mice was longer compared to PBS treated mice ($p < 0.05$; Fig. 1G).

Jointly, these results indicate that long-term memory is impaired in adult mice subjected to moderate neonatal HI and intranasal C3a treatment is protective against such cognitive impairment.

Exploratory behavior and locomotion are not altered by HI injury or C3a treatment

To confirm that HI or C3a treatment did not affect general exploratory behavior or locomotion of mice, we used an open field evaluation of sham mice treated with PBS and mice subjected to HI and treated with PBS or C3a for 3 days. Mice showed comparable average speed, activity levels, locomotion time and distance travelled in all three groups at P50, indicating that HI or C3a treatment did not affect general exploratory and locomotor activities (Fig. 2A-D). Furthermore, there were no differences between groups regarding the distance travelled and time spent in the center of the arena or the number of rearing and grooming episodes, indicating that anxiety-like behavior was not induced by HI or C3a treatment (Fig. 2E-H).

Intranasal C3a ameliorates HI-induced glial response

To determine the effect of C3a treatment on glial responses, we quantified GFAP and Iba-1⁺ immunoreactivity in the CA / DG of the dorsal hippocampus at P55 in mice treated for 3 days after HI. We found that HI on P9 led to an increase in GFAP⁺ area in the ipsilateral compared with contralateral CA and DG regions in PBS treated mice, indicating HI-induced astrocyte activation. In both CA1 and CA3, this increase was also apparent in comparison with sections from sham mice. The proportion of GFAP⁺ relative area in CA1 and DG of C3a treated mice did not differ between the hemispheres or in comparison with sham mice (Fig. 3B). C3a treatment did not affect HI-induced astrocyte activation in CA3 (Fig. 3B). Mice subjected to HI and administered PBS exhibited higher density of Iba-1⁺ cells in the

ipsilateral CA and DG compared to contralateral CA and DG, indicating HI-induced microglial proliferation. In C3a treated mice, the density of Iba-1⁺ cells did not differ between the hemispheres (Fig. 3C). Jointly, these results provide the evidence for ameliorated response of astrocytes and microglia in the C3a treated mice.

Intranasal C3a does not affect HI-induced loss of hippocampal tissue

Consistent with our previous report (Järlestedt et al., 2013), moderate HI at P9 resulted in more than 50% reduction in the volume of the hippocampus in the injured compared to contralesional hemisphere at P51-55 ($p < 0.001$). Intranasal C3a administration did not affect HI-induced loss of hippocampal tissue (Fig. 4).

To assess potential adverse effects of intranasal C3a administration on the overall animal health status and development, we recorded the animal weight gain across surgeries and treatments. The results show that neither the surgery nor the C3a treatment affected body weight gain in mice (Fig. S1), thus excluding any pronounced adverse effect of the C3a treatment. Together, these results demonstrate that the intranasal treatment of mice with C3a is safe but does not have any measurable neuroprotective or growth stimulatory effect in the HI injured hippocampus.

Intranasal C3a does not affect HI-induced decrease in neuronal density in the dorsal hippocampus

Next, to determine the effect of C3a treatment on neuronal density, we quantified NeuN⁺ cells in the CA / DG of the dorsal hippocampus at P55 in mice

treated for 3 days following HI. We found that in CA1 and CA3, neuronal density was decreased in the ipsilesional hemisphere compared with contralesional hemisphere of both HI groups ($p<0.01$ and $p<0.05$, respectively, Fig. 5). The number of NeuN⁺ cells of the injured CA1 of PBS but not C3a treated mice was lower compared with sham mice ($p<0.05$, Fig. 5B). In the injured CA3 of C3a but not PBS treated mice, the numbers of NeuN⁺ cells were lower compared with sham mice ($p<0.01$, Fig. 5C). No effect of HI or C3a treatment was found with regard to NeuN⁺ cell density in the DG (data not shown). Jointly, these results demonstrate that neonatal HI leads to a substantial reduction in neuronal density in the CA of the dorsal hippocampus, this neuronal loss is not substantially affected by intranasal C3a treatment.

HI leads to the increase in GAP-43 expression in the dorsal hippocampus that is not affected by C3a treatment

Next, we performed immunostaining with antibodies against GAP-43, a phosphoprotein localized in the neuronal growth cone that is involved in neurite extension and regarded as a surrogate marker of axonal plasticity (Benowitz and Routtenberg, 1997); it also mediates glial plasticity during astrogliosis (Hung et al., 2016). GAP-43 expression is highly upregulated in the adult cortex after ischemic stroke (Carmichael et al., 2005). We did not find any differences between groups with regard to the density of GAP-43⁺ puncta in the CA1 (Fig. 5D). However, the average GAP-43⁺ punctum area in the CA1 of the HI injured hippocampus of PBS treated, but not C3a treated, mice was larger compared with sham mice ($p<0.05$, Fig. 5E). No effect of HI or C3a treatment was found with regard to the expression

of GAP-43 in the dentate gyrus (Fig. S2A-B). These data show that in response to HI, the different regions of the dorsal hippocampus respond differently to HI with regard to the expression of GAP-43.

We also used antibodies against synapsin I (a pan-synaptic marker, (Micheva et al., 2010)) and VGLUT1 that has been shown to visualize the majority of glutamatergic synapses (Micheva et al., 2010). We did not find any differences in the CA1 (Fig. 6) or dentate gyrus (Fig. S2) of the dorsal hippocampus in the density or average area of synapsin I⁺ and VGLUT1⁺ puncta between the groups or between the HI injured and contralesional hemisphere. As the VGLUT1 positive synapses stand for approximately 75% of all synapses visualized by antibodies against synapsin I and quantified at array tomography level (Micheva et al., 2010), the higher density of VGLUT1 positive puncta as compared to the density of puncta visualized by the antibody against synapsin I in our study conceivably reflect the difference in affinity and detection threshold between the antibodies used.

Discussion

The most important finding of our study is that a short-term treatment with C3a administered in a non-invasive and clinically feasible manner ameliorates HI-induced long-term memory impairment. Intranasal C3a treatment ameliorated reactive gliosis in the CA1 and DG but did not reverse HI-induced hippocampal tissue loss / atrophy or reduction in neuronal density. Likewise, axonal sprouting and synaptic density, as assessed by the expression of GAP-43, and synapsin I together with VGLUT1 immunohistochemistry, respectively, in the hippocampus were not affected by intranasal C3a treatment. Given that systemic C3a receptor activation can cause hypersensitivity or anaphylaxis due to histamine release from basophiles and mast cells (Johnson et al., 1975), it is noteworthy that intranasal administration of C3a peptide is associated with a minute general response to intranasal administration of a non-sensitizing agent and does not lead to any adverse systemic response (Stokowska et al., 2016).

ORT takes advantage of the innate ability of a rodent to remember a familiar object and their spending more time exploring a novel object than a familiar object. Performance in this test depends on normal function of the hippocampus (Clark et al., 2000) and the perirhinal cortex (Winters and Bussey, 2005). We observed that although the PBS treated mice spent more time exploring the novel object when tested 6 h after the memory formation, they did no longer discriminate between the familial and novel object when tested the day after. In contrast, the sham mice and C3a treated mice spent more time exploring the novel object at both time points. Other investigators using this test showed that severe neonatal HI (45-60 min exposure to 10% O₂) leads to the loss of very short (5 min) (Donega et al., 2015) to short-term (1 h) (McAuliffe et al., 2009) memory of the familial object. Our results

demonstrate that long-term memory is impaired by milder HI insult (30 min exposure to 10% O₂) and that this cognitive impairment can be reversed by intranasal C3a treatment. Notably, whereas neonatal HI did not alter the total exploration time at any of the testing occasions, C3a treatment post-HI increased the total exploration time in comparison with PBS-treated HI mice, but only in the presence of the novel object. In the absence of any effects of C3a treatment on the expression of GAP-43 and the pre-synaptic markers, the underlying mechanisms remain elusive and warrant further investigation of the effects of C3a in the injured neonatal brain.

Our findings that neither neonatal HI nor C3a treatment changed locomotive and exploratory behavior of mice are in line with a previous report that only severe HI leading to cyst formation altered behavior in an open-field test in comparison with naïve animals (Ten et al., 2004). In accord with previous study (Reinboth et al., 2016), our data further show that unilateral HI injury does not induce anxiety-like behavior.

While over-expression of C3a in reactive astrocytes was beneficial in terms of tissue protection, single dose intraventricular administration of C3a 1 h after HI induction was not neuroprotective (Järlestedt et al., 2013). The single dose C3a treatment did, however, prevent HI-induced cognitive impairment in wild-type mice as assessed 42 days later (Järlestedt et al., 2013). These results, together with the present data suggest that the reversal of HI-induced cognitive impairment by C3a is independent of neuronal density. Similarly, estradiol therapy improved behavioral performance but did not reverse the HI-induced loss of hippocampal volume (Waddell et al., 2016). Adaptive neural plasticity responses that improve neuronal functioning thus may play a critical role in functional outcome after neonatal

ischemic injury. In support of this notion, exposure to enriched environment was effective in recovering declarative but not aversive memory impairment and preserved hippocampal dendritic spine density loss after neonatal HI injury in rats (Rojas et al., 2013). Environmental enrichment, however, did not affect HI-induced tissue atrophy in the hippocampus (Pereira et al., 2008).

Intranasal C3a treatment starting 7 days after ischemic injury to the adult brain led to faster and sustained recovery of motor function associated with increased expression of GAP-43, synapsin I and VGLUT1 (Stokowska et al., 2016). Similar to the peri-infarct region after ischemic stroke (Stokowska et al., 2016), we observed that HI led to increased expression of GAP-43 in the hippocampal CA1 but the GAP-43 expression was not further increased by C3a treatment. The expression of pre-synaptic markers was not affected by HI or C3a treatment. Thus, in contrast to ischemic injury to the adult brain, the positive effects of intranasal C3a on cognitive performance of mice do not appear to be mediated via increased expression of markers of spouting axons or synapses but rather involve other components of neuronal functioning such as modulation of synaptic function. In support of this notion, in vitro, C3a increased neurite outgrowth of newly differentiated neurons (Shinjyo et al., 2009) and basal C3aR signaling has been suggested to play a role in normal dendritic extension (Lian et al., 2015). In addition, C3aR signaling was shown to increase synaptic strength by promoting membrane localization of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Lian et al., 2015). In adult mice, single intraventricular injection of 10 pmol C3a 30 min prior to training session was reported to ameliorate amnesia induced by global cerebral ischemia when assessed 24 h later, although the underlying mechanisms were not addressed by the authors (Jinsmaa et al., 2000).

Our findings of the reversal of HI-induced cognitive impairment by intranasal C3a are in line with these previous reports and provide a strong evidence for the role of C3a in protection against injury-induced amnesia. The anti-amnestic effect of C3a observed after brief intranasal treatment with the peptide in the absence of any detectable adverse effects of the treatment provides direct evidence that targeting C3aR in the injured immature brain could be clinically feasible and potentially applicable to the treatment of infants at high risk of developing HIE.

Consistent with previous reports (Chavez-Valdez et al., 2012; Qiu et al., 2007; Teo et al., 2015) we observed HI-induced astrocyte activation in the ipsilateral hippocampus. This astroglial response, which persists for at least 6 weeks after HI, was ameliorated by intranasal C3a treatment in a region specific manner. These findings suggest that the beneficial effects of intranasal C3a treatment in terms of cognitive performance may be mediated, at least partially, by astrocytes. This reasoning is further supported by our previous data demonstrating that C3a attenuated ischemia-induced upregulation of GFAP in cultured primary astrocytes and increased astrocyte survival after ischemic stress (Shinjo et al., 2016). Notably, constitutive absence of GFAP in combination with the absence of vimentin, another intermediate filament protein required for the formation of intermediate filaments in reactive astrocytes (Pekny and Pekna, 2014, 2016), did not affect tissue loss after HI (Järlestedt et al., 2010). However, similar to C3a over-expression in reactive astrocytes, the absence of GFAP and vimentin increased the numbers of surviving neurons newly born after HI (Järlestedt et al., 2010). Studies in GFAP deficient mice may shed light on the specific role of GFAP on HI-induced cognitive impairment. Our findings that C3a treatment reduced HI-induced GFAP upregulation in CA1 but not in CA3 point to differential glial responses in these CA

regions. Profound region-specific differences in C3-mediated age-dependent elimination of hippocampal CA synapses were recently reported, although the investigators did not address the role of reactive gliosis in this process (Shi et al., 2015). The mechanisms underlying CA region specific effects of C3a on astrocyte activation and the potentially distinct roles of C3b and C3a, the two products of C3 activation, in the hippocampus merit further investigation. It is noteworthy that astrocytes are involved in activity-dependent synapse remodeling in the developing and adult brain (Chung et al., 2013). Although it remains to be determined whether astrocyte-mediated synapse remodeling underlies learning and memory, it is conceivable that the synapse remodeling function of astrocytes is altered when astrocytes respond ischemic insults.

Previous studies found increased density of microglial cells in the ipsilateral hippocampus of juvenile mice 7 to 9 days post-HI (Cikla et al., 2016; Jantzie et al., 2005). Our data demonstrate that this HI-induced response of microglia is evident even after the mice reached adulthood and can be attenuated by intranasal C3a treatment. In the absence of any measurable effect of C3a treatment on neuronal density, these findings further support the contention that the effects of C3a on neuronal function are conceivably mediated through the microglial compartment. Microglia play a wide range of functions in the healthy and diseased brain (Kettenmann et al., 2013). Microglia eliminate excessive synapses during development in a C3b-complement receptor 3 (CR3) dependent manner (Schafer et al., 2012), microglial CR3 activation triggers long-term synaptic depression in the hippocampus (Zhang et al., 2014). Microglia protect the neonatal brain from hemorrhage after acute ischemic stroke (Fernandez-Lopez et al., 2016) and contribute to neuroprotection during the subacute injury phase (Faustino et al.,

2011). Under physiological conditions, microglia promote learning-dependent synapse formation (Parkhurst et al., 2013). C3a directly affects intracellular signaling (Möller et al., 1997) and induces NGF expression in microglia (Heese et al., 1998). Acute C3a treatment promotes, whereas chronic C3/C3a treatment attenuates, microglial phagocytosis (Lian et al., 2016). Better understanding of microglial responses following neonatal HI, as well as the microglia-specific mechanisms underlying the beneficial effects of intranasal C3a on the neonatal brain in the context of HI injury, warrant further investigation.

In summary, our results demonstrate that a brief pharmacological treatment with C3a using a clinically feasible non-invasive mode of administration can reverse HI-induced cognitive impairment, possibly through targeting the glial compartment. Intranasal administration appears to be a plausible route to deliver C3a into the brain of asphyxiated infants at high risk of developing HIE. Further studies will determine the effects of intranasal C3a on brain development and cognitive performance of naïve mice.

Conflict of Interest

The authors declare that they have no conflict of interest.

Figure legends

Fig. 1. Intranasal C3a treatment ameliorates hypoxia-ischemia induced cognitive impairment

At P9, mice were subjected to sham procedure or hypoxia-ischemia followed by intranasal treatment with PBS or C3a for 3 days. (A) Schematic of the study design. Locomotive behavior and memory were assessed by open field and object recognition test, respectively. Time spent exploring individual objects and total exploration time during familiarization (B, C), short-term memory testing 6 h later (D, E) and long-term-memory testing 24 h later (F, G). A, A'=identical objects used during the familiarization phase; n=8-11 per group; mean \pm SEM. HI, hypoxic-ischemic injury. # $p < 0.05$, ### $p < 0.001$ novel vs. familiar object; * $p < 0.05$, ** $p < 0.01$ C3a vs. PBS. Statistics used were two-way ANOVA and one-way ANOVA for evaluating the time spent exploring individual objects and total exploration time, respectively, followed by a Tukey's multiple comparisons post-hoc test.

Fig. 2. Locomotion and activity in open field are not altered by hypoxic-ischemic injury or C3a treatment

On P50, locomotion and general exploratory activity were assessed in open field. (A) Average speed, (B) total activity, (C) total locomotion time, (D) total distance travelled, (E) distance travelled in the center, (F) time spent in the center, (G) number of rearings, (H) and number of grooming episodes. n=18-19 per group; mean \pm SEM. HI, hypoxic-ischemic injury.

Fig. 3. Intranasal C3a ameliorates hypoxia-ischemia–induced reactive gliosis

Representative images of ipsilesional CA1 region of the dorsal hippocampus immunostained with anti-GFAP and anti-Iba-1 antibody, respectively, from P55 mice subjected to sham procedure or hypoxia-ischemia on P9 and treated with intranasal PBS or C3a for 3 days (A). (B) GFAP positive area relative to total area of CA1, CA3 and dentate gyrus (DG). (C) Density of Iba-1 positive cells in CA1, CA3 and dentate gyrus (DG). Mean \pm SEM. (n=6 per group). # $p<0.05$, ## $p<0.01$, ### $p<0.001$ ipsilesional vs. contralesional; * $p<0.05$, *** $p<0.001$ HI vs. sham by two-way ANOVA and Tukey's multiple comparisons post-hoc test. HI, hypoxic-ischemic injury. GFAP, glial fibrillary acidic protein. Iba-1, ionized calcium-binding adapter molecule 1. Scale bar=50 μ m.

Fig. 4. Intranasal C3a does not affect hypoxia-ischemia–induced loss of hippocampal tissue

Representative images of hematoxylin-eosin stained coronal brain sections from each group at -1.80 mm distance from Bregma (A). (B) Volume of hippocampus relative to hemisphere volume of P55 mice subjected to sham procedure or hypoxia-ischemia on P9 and treated with intranasal PBS or C3a for 3 days (n=14-15 per group). Mean \pm SEM. ### $p<0.001$ ipsilesional vs. contralesional; *** $p<0.001$ HI vs. sham by two-way ANOVA and Tukey's multiple comparisons post-hoc test. HI, hypoxic-ischemic injury; CL, contralesional; IL, ipsilesional. Scale bar=1mm.

Fig. 5. Hypoxia-ischemia decreases neuronal density and leads to an increase of GAP-43 expression in the injured dorsal hippocampus

Representative images of NeuN positive cells (ipsilesional dorsal hippocampus, scale bar=200 μ m) and GAP-43 positive puncta (ipsilesional CA1 region of the dorsal hippocampus, scale bar=10 μ m) from P55 mice subjected to sham procedure or hypoxia-ischemia on P9 and treated with intranasal PBS or C3a for 3 days (A). Density of NeuN positive cells in CA1 (B) and CA3 (C) regions of the dorsal hippocampus. (D) Density of GAP-43 positive puncta and (E) mean area of GAP-43 positive puncta in CA1 (n=6 per group). Mean \pm SEM. # $p<0.05$, ## $p<0.01$ ipsilesional vs. contralesional; * $p<0.05$, ** $p<0.01$ HI vs. sham by two-way ANOVA and Tukey's multiple comparisons post-hoc test. HI, hypoxic-ischemic injury. NeuN, neuronal nuclei. GAP-43, growth associated protein 43.

Fig. 6. Hypoxia-ischemia does not affect the overall synaptic density or the density of glutamatergic synapses in the injured dorsal hippocampus

Representative images of synapsin I and VGLUT1 positive puncta in the ipsilesional CA1 region of the dorsal hippocampus from P55 mice subjected to sham procedure or hypoxia-ischemia on P9 and treated with intranasal PBS or C3a for 3 days (A). (B) Density of synapsin positive puncta. (C) Mean area of synapsin I positive puncta. (D) Density of VGLUT1 positive puncta. (E) Mean area of VGLUT1 positive puncta (n=6 per group). Mean \pm SEM. HI, hypoxic-ischemic injury; SYN, synapsin I; VGLUT1, vesicular glutamate transporter 1; scale bar=10 μ m.

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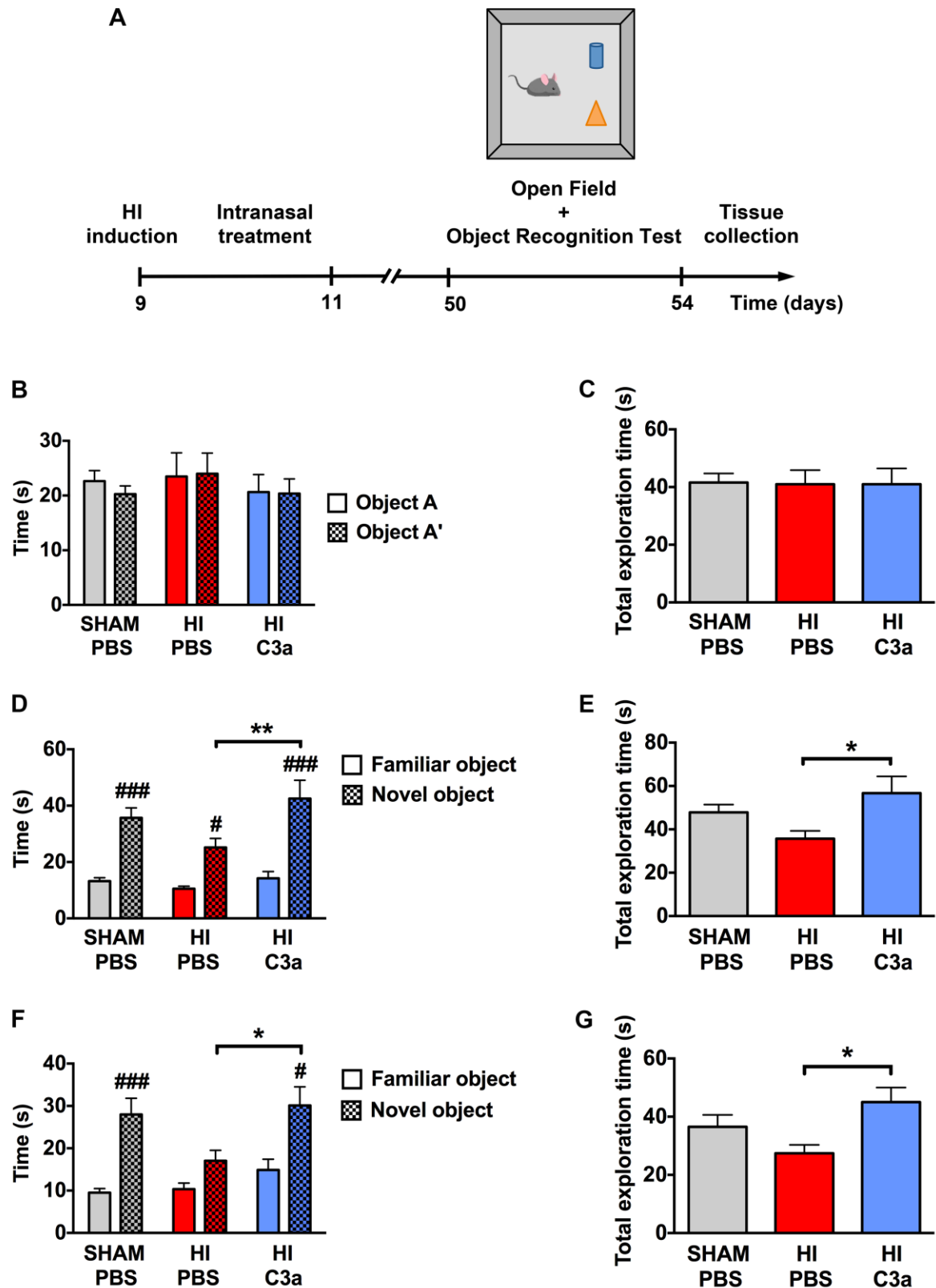


Fig. 1

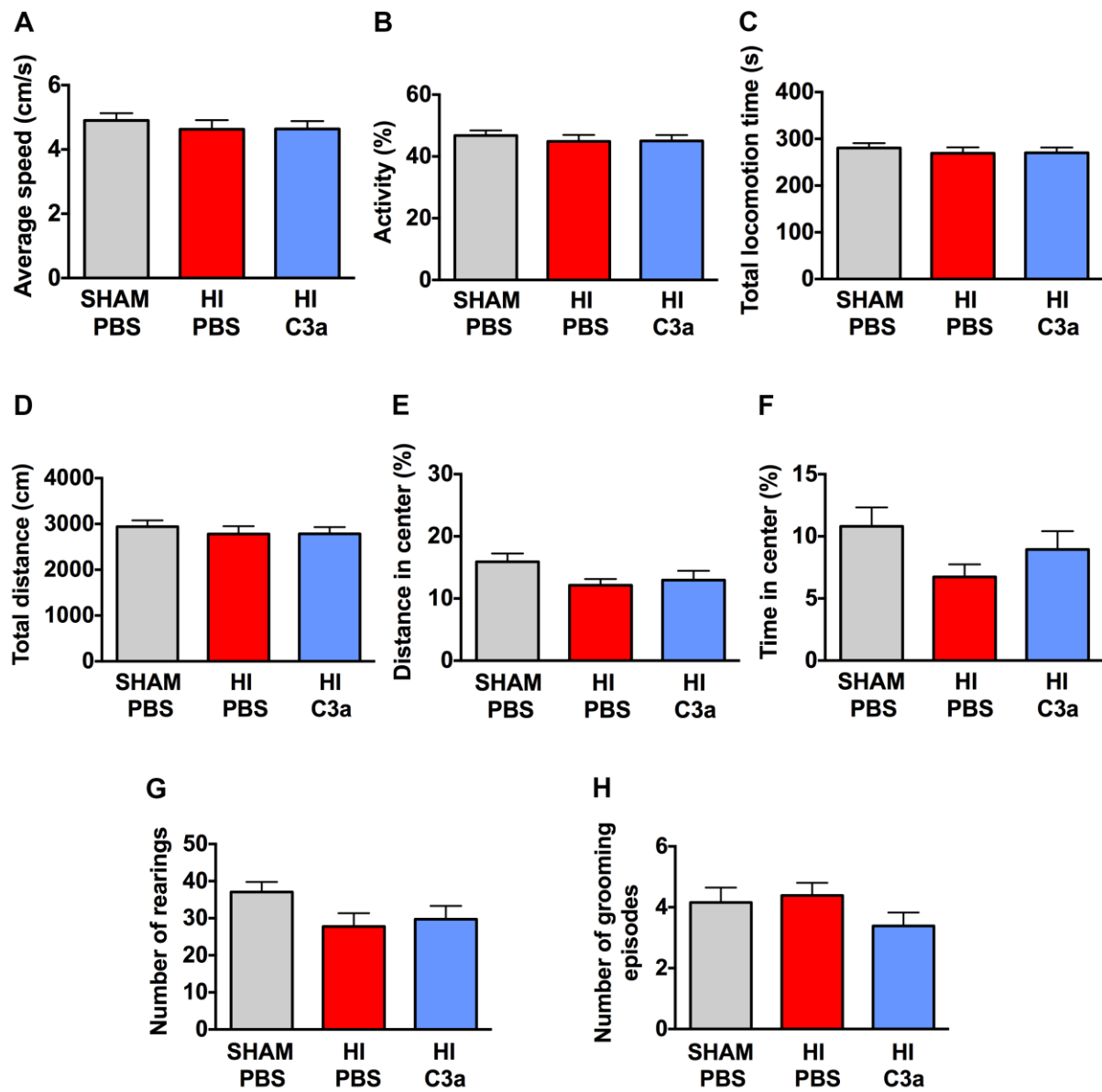


Fig. 2

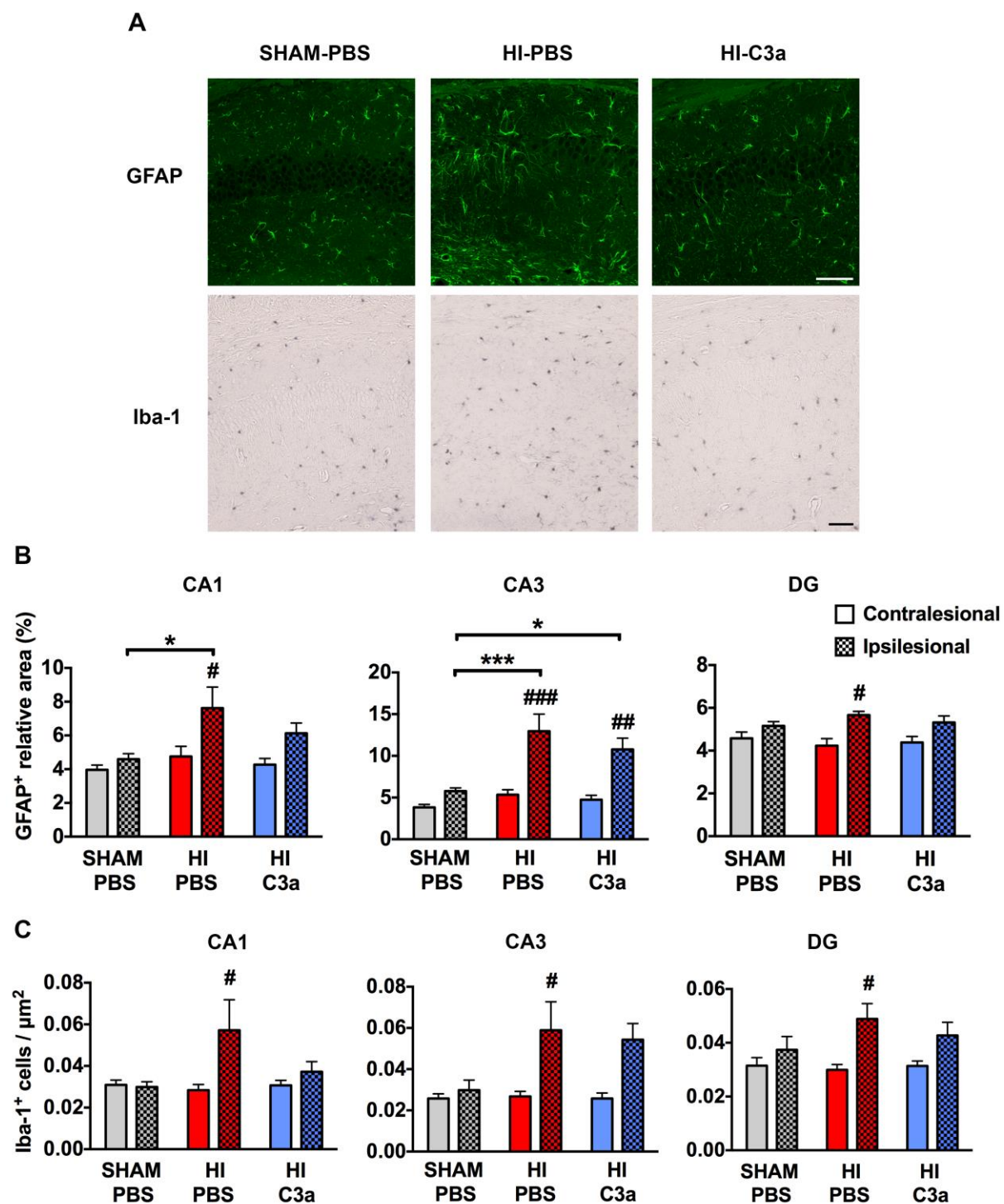


Fig. 3

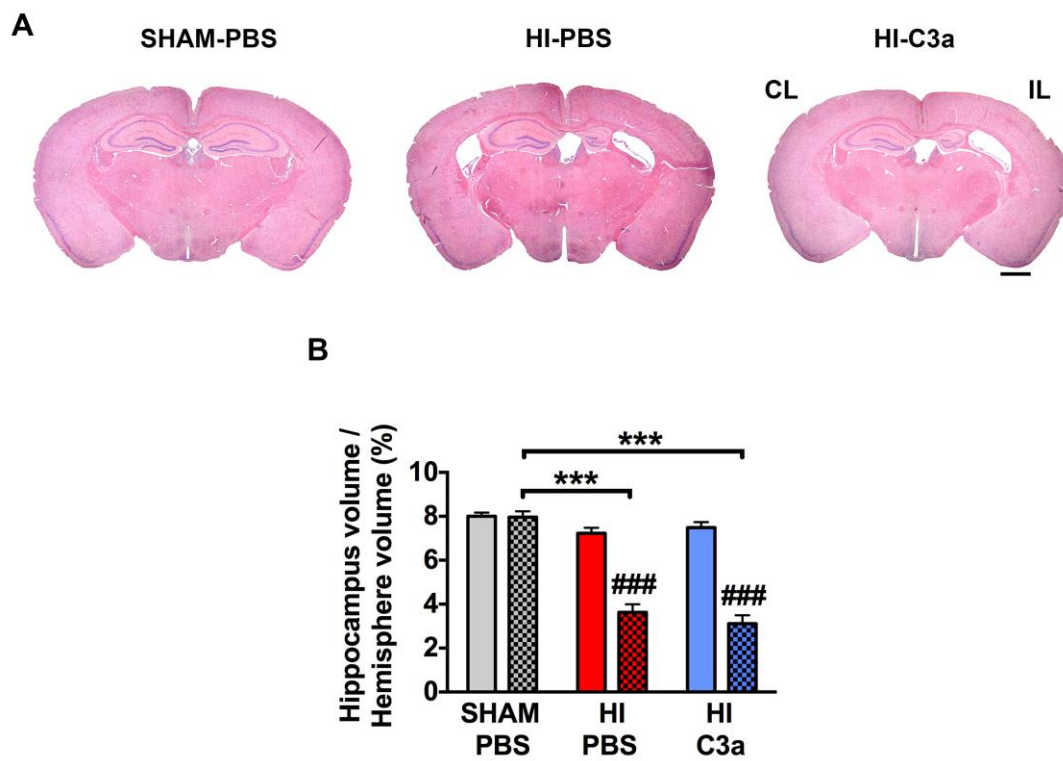


Fig. 4

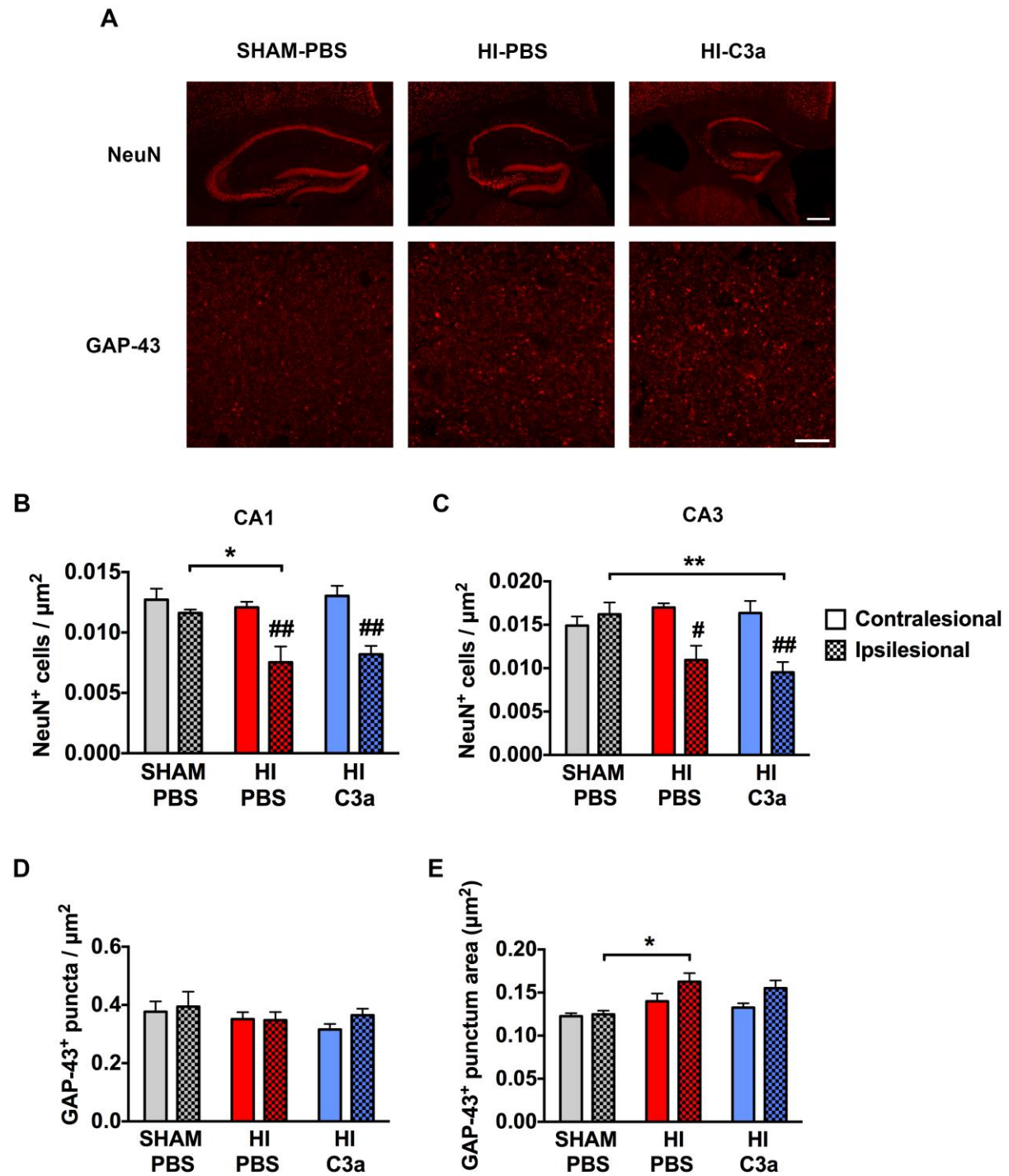


Fig. 5

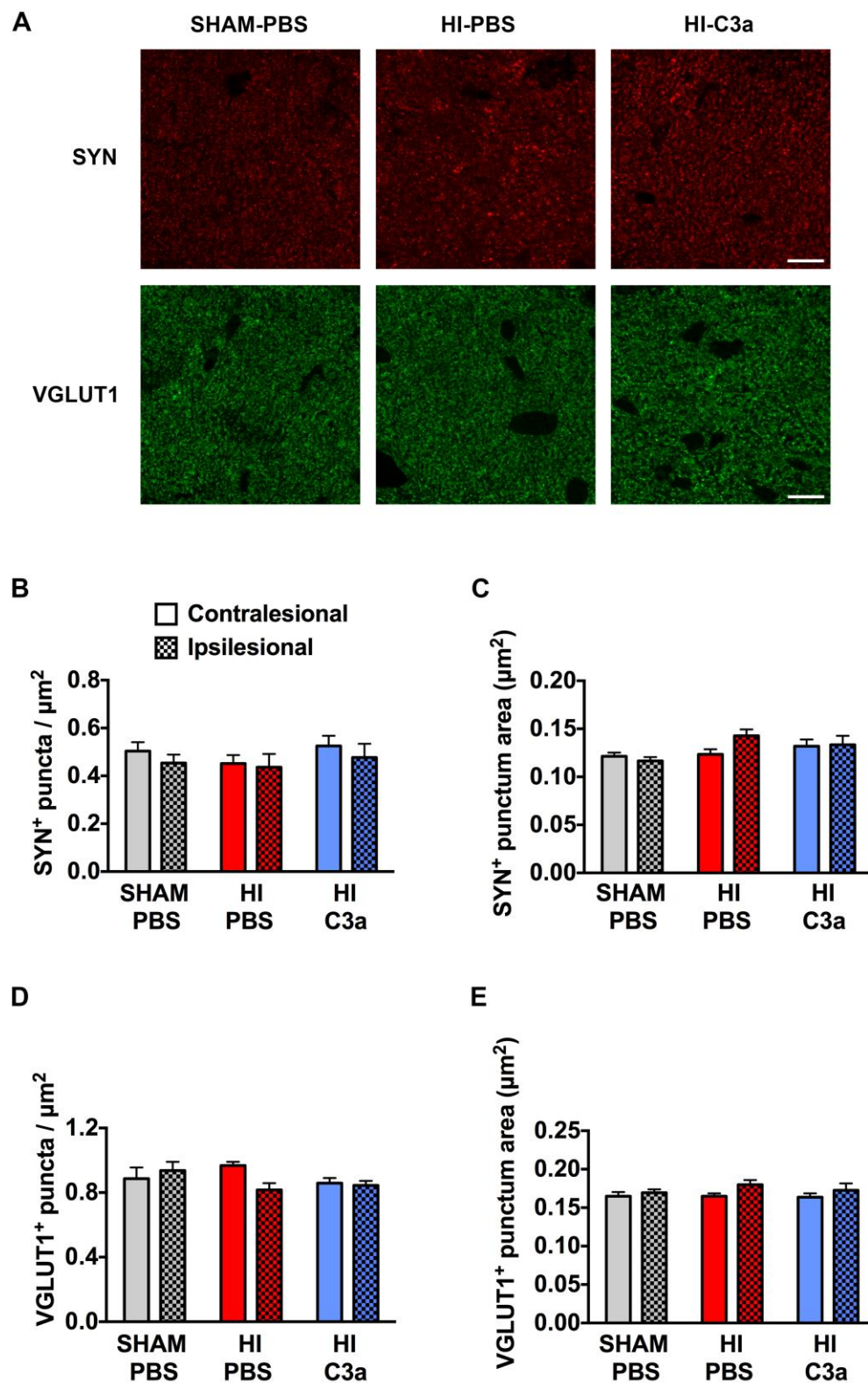
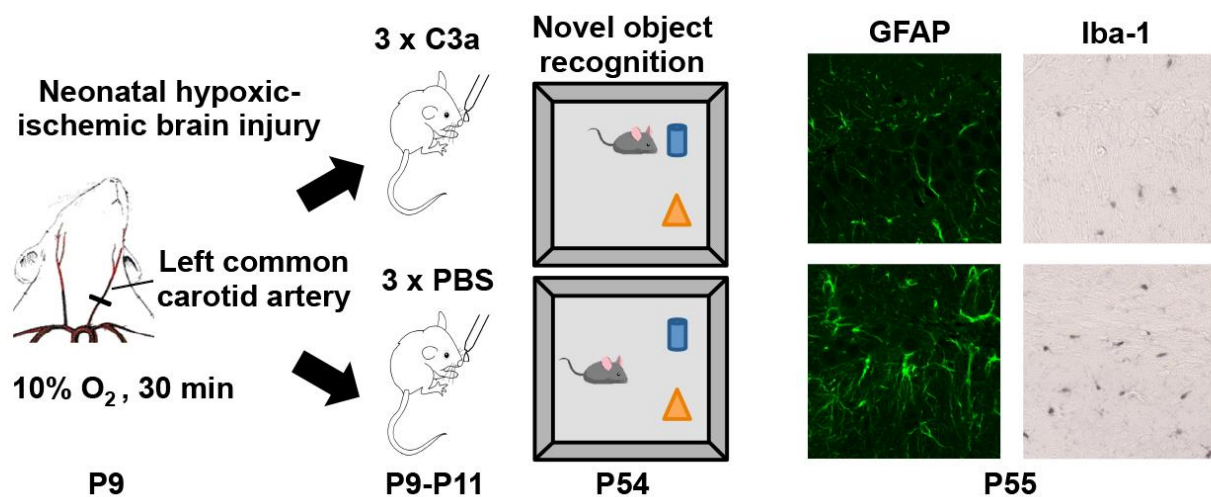


Fig. 6



Graphical abstract

Highlights

- Intranasal C3a ameliorated hypoxia-ischemia (HI)-induced cognitive impairment
- Exploratory behavior and locomotion were not altered by HI or C3a treatment
- Intranasal C3a ameliorated HI-induced reactive gliosis
- Intranasal delivery of C3a may be a therapeutic strategy for birth asphyxia